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AUTHOR(S):

Tanabe, Reiko

CITATION:

Tanabe, Reiko. Inactivation of a-Amylase in Aqueous Solution by Gamma Ray Irradiation (Special Issue on Physical, Chemical and Biological Effect of Gamma Radiation, XIV). Bulletin of the Institute for Chemical Research, Kyoto University 1973, 51(1): 31-36

ISSUE DATE:

1973-03-31

URL:

http://hdl.handle.net/2433/76472

Bull. Inst. Chem. Res., Kyoto Univ., Vol. 51, No. 1, 1973

Inactivation of α-Amylase in Aqueous Solution by Gamma Ray Irradiation

Reiko TANABE*

Received January 16, 1973

The effects of γ-ray irradiation on Bacterial α-amylase in aqueous solution were studied. Inactivation of α-amylase by irradiation was caused by rather indirect action than direct one of radiation. Among the various species of radiolyzates from water, H_2O_2 was not so effective, but OH radicals were very effective to inactivation of this enzyme. Optimum pH of the irradiated enzyme in enzyme reaction was similar to that of intact enzyme.

INTRODUCTION

Studies on the inactivation or activation of enzymes by irradiation have been investigated by a number of groups of workers. But the great majority of these studies has dealt with the inactivation of enzyme which has SH groups or S–S bonds in its molecule, such as alcohol dehydrogenase, glutamic dehydrogenase, RNase and so on. From the results of studies of these enzymes, it was proposed that the inactivation of the enzyme by irradiation was caused by a damage of SH-groups or S–S bonds.[1] But the high sensitivity or specificity of these loci in accepting the irradiation effects may make impossible to estimate the function of other amino acid residues or protein comformation in relation to the enzyme activity. On the one hand, in the case of enzyme which has neither SH groups nor S–S bonds in its molecule, the studies of its inactivation mechanism by irradiation should be generalized and be more suitable to presume the essential elements to enzyme activity. The present studies deal with the mechanism of inactivation of enzyme which has nor SH-groups nor S–S bonds. The enzyme used here is named bacterial α-amylase, which is produced by BACILLUS SUBTILIS. It has been reported by Junge[2] and Akabori *et al.*[3] that his enzyme has nor SH groups nor S–S bonds in its molecular structure.

In this paper, it is confirmed that the inactivation of α-amylase in aqueous solution by irradiation is caused by indirect action attributed to the reaction between the enzyme molecule and radiolyzates from water such as OH·, O_2H, H·, H_2O_2, and so on. The results from experiments to investigate the most effective species of those radiolyzates from water for inactivation of the enzyme are also described.

MATERIALS AND METHODS

Bacillus subtilis α-amylase used in this work was a crystalline preparation made from the culture of BACILLUS SUBTILIS DT–57 strain by Daiwa Kasei Co. Ltd., Osaka and was used without further purification. This preparation contains calcium acetate in about 40 percent as a stabilizer. Its molecular weight is about 48,000 and it is rich

* 田辺レイ子: Depatment of Chemistry, Faculty of Science, Kyoto University, Kyoto.

in neutral and acidic amino acids such as aspartic acid, glutamic acid, glysine, and so on as shown in the following paper.

Soluble starch used as a substrate in the enzyme reaction was obtained from Nakarai Chemical Co. Ltd. Kyoto.

α-Amylase activity was determined by measuring the amount of aldehyde groups of hydrocarbon liberated by enzyme action according to Nelson's method[4] calorimetrically using an EPU-2, Hitachi photoelectric spectrophotometer. The enzyme reaction was started by adding 1 ml of enzyme solution (2 μg/ml) to a reaction mixture consisted with 2 ml of 0.5 percent soluble starch and 1 ml of 0.1 M acetate buffer, pH 6.0. After the incubation for 10 min at 40°C, the enzyme reaction was interrupted by adding 2 ml of the Somogyies deproteinizing reagent. After filtered, an increase of the reducing power in this filtrated reaction mixture was measured calorimetrically. The optical density observed in this assay was expressed as the amount of D-glucose which was calculated from the standard curve of D-glucose by Nelson's method.

γ-ray irradiations were carried out using two apparatuses. One was 2000 Ci ^{60}Co Irradiation Facility with a dose rate of 1.2×10^5 r/hr in the Institute for Chemical Research, Kyoto University, and the other was a Toshiba's RE1010 100 Ci ^{60}Co Irradiation Apparatus[5] with the dose rate of 4.63×10^4 r/hr in our laboratory. α-Amylase was dissolved in doubly distilled water saturated with air and its concentration was 0.1 percent in most cases. α-Amylase solution was irradiated in a 17 mm diameter hardglass tube at room temperature.

Irradiation of ultraviolet lights to 0.1 percent α-amylase solution containing various ancounts of H_2O_2 was carried out in 3 mm diameter quartz capillary by a 100 W mercury lamp at a distance of 10 cm without any filter.

RESULTS

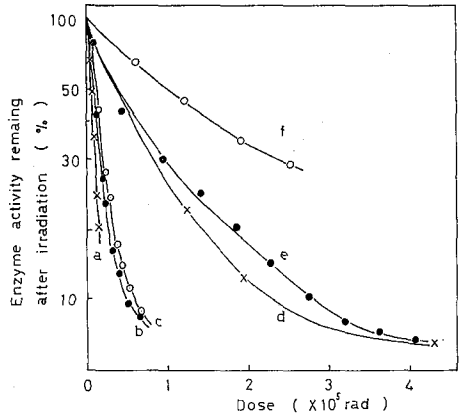

Fig. 1-a : Effect of γ-rays on α-amylase activity.
Curve a : 92 μg/ml, b : 250 μg/ml, c : 1.44 mg/ml, d : 2.50 mg/ml, e : 3.40 mg/ml, and f : 6.40 mg/ml enzyme solu tion.

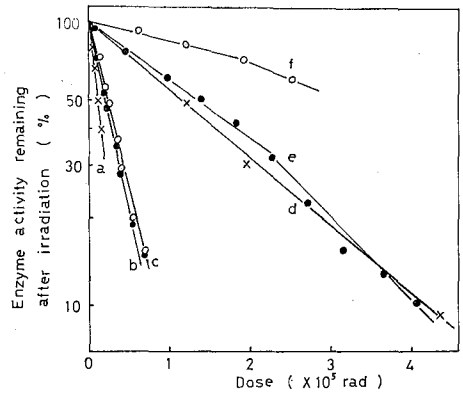

Fig. 1-b : Effect of γ-rays on α-amylase activity.
The ratio of remaining activity of α-amylase to drown in a logarithmic scale.
Symbols, a, b, c, d, e and f show the same concentrations of enzyme solution as those of Fig. 1-a.

Enzyme inactivation by γ-ray irradiation

When the enzyme solutions of various concentration were exposed to γ-ray irradiation, their activity decreased as shown in Fig. 1-a. The more the concentration of enzyme solution increases, the greater the irradiation dose required for the inactivation of enzyme becomes. For example, the dose required to reduce the remaining activity ratio to 37 percent, D_{37}, is 1×10^4 rad for 92 μg/ml enzyme solution, 2.4×10^4 rad for 1.44 mg/ml enzyme solution, and 1.2×10^5 rad for 2.5 mg/ml enzyme solution. The inactivation of enzyme proceeded exponentially as shown in Fig. 1-a and these data more or less closely fit straight lines, when the remaining activity is plotted versus the irradiated dose semilogarithmically. This plotting was shown in Fig. 1-b. The extrapolations to the vertical gave a value of about 1.3, and this fact showed that the inactivation type of this enzyme was the one hit type.

Concentration effect

When the effect of irradiation on enzyme in aqueous solution, not in dry state, is studied, it seems necessary to investigate whether the inactivation of enzyme is caused by the direct action or by the indirect action of radiation. The influence of the concentration of enzyme to the enzyme inactivation was investigated. The numbers of the inactivated enzyme obtained from the data at the dose of 2×10^3 rad in Fig. 1-a were plotted versus the concentration of α-amylase in the solution. The result is shown

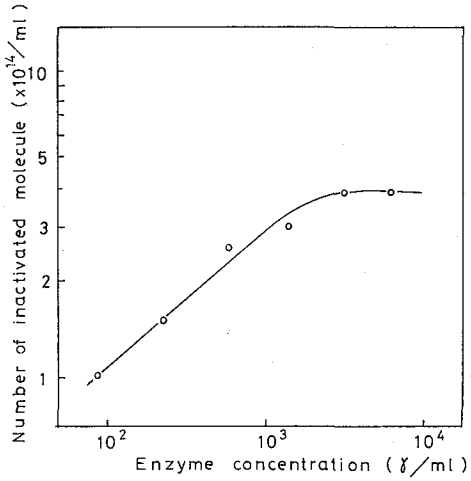

Fig. 2. Reaction yields of the inactivation of α-amylase in various concentration of solutions at the dose of 2×10^3 rad. Reaction yields are described as G-value.

in Fig. 2. The numbers of enzyme molecule were calculated on the basis where the molecular weight of this enzyme protein was 48,000. In the region where the enzyme concentration is higher than 10^{-3} g/ml, G-values of the enzyme inactivation took a definite value and this fact showed the presence of concentration effect in the inactivation by irradiation. The number of inactivated enzymes did not depend on enzyme concentration, but on the irradiation dose which determines the amount of radicals originated in solvent water. The decrease of G-value shown in the lower part of the

enzyme concentration, may be attributed to the reconbination of radicals.

Effective species of radiolyzates from water for the enzyme inactivation

When the inactivation of enzyme is caused by indirect action of radiation, it should be occured by radiolytic substances or radicals from water. The effects of H_2O_2 on enzym activity were investigated by the method as follows. After the mixtures of α-amylase and H_2O_2, in which the enzyme concentration was 0.1 percent and H_2O_2 is in various concentration, were incubated for 20 minutes at room temperature, the enzyme activity in these mixtures was measured by the method described in METHODS. As shown in Fig. 3, the enzyme activity was decreased only when the concentration of H_2O_2 was higher than 10^{-2} M. The free radical, OH, can be produced chemically or photochemically as reported by B. Holmes *et al.*[6]

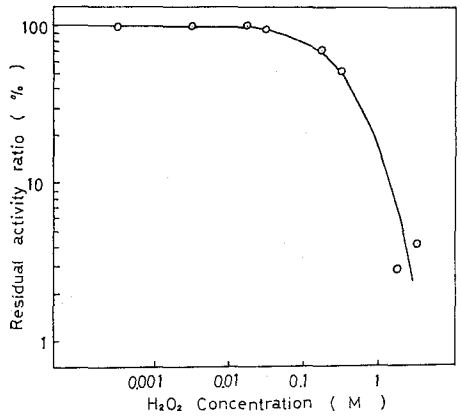

Fig. 3. Effect of H_2O_2 on α-amylase activity.

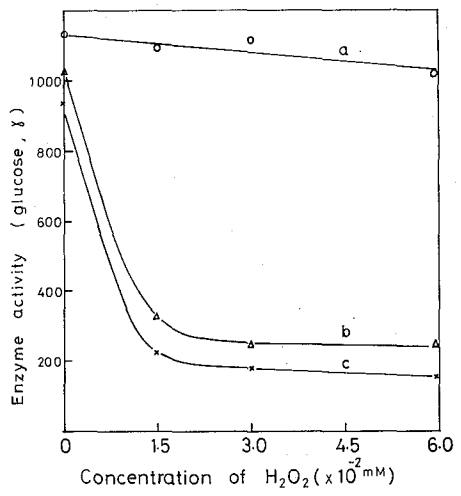

Fig. 4. Effect of OH radicals on α-amylase. Remaining activity ratio was calculated against the activity of intact enzyme which was not treated with H_2O_2 nor UV irradiation.
Curve a : none irradiated, b : irradiated for 1 minute and c : for 2 minutes.

The effect of OH radicals produced by the Fenton's reagent was investigated. But this enzyme activity was inhibitted strongly by ferric ion and this attempt was unsuccessful. OH radicals were produced by an irradiation of UV lights in the presence of H_2O_2, and its effect on the enzyme activity was investigated by the method as described above. Figure 4 indicates that either addition of H_2O_2 or irradiation of UV lights in the absence of H_2O_2 causes only a little inactivation, but that OH radicals inactivate the enzyme immediately.

After effect for the irradiated enzyme

After 0.1 per cent α-amylase solutions were irradiated with various doses of γ-

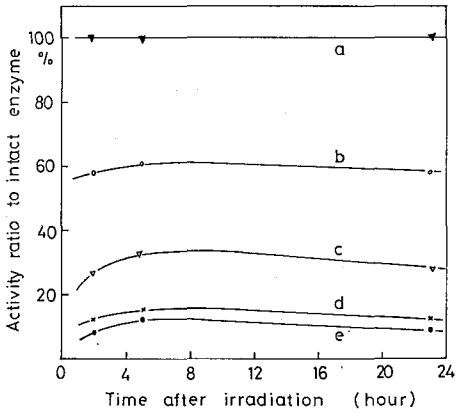

Fig. 5. Activity of inactivated enzyme after incubation at 4°C.
The concentration of the enzyme solutions in this incubation was 0.1per cent.
Curve a : none irradiated, b : irradiated by 2.7×10^4 rad, c : by 5.7×10^4 rad, d : by 2.7×10^5 rad, and e : by 5.7×10^5 rad.

rays and incubated at 4°C for several hours, the enzyme activity in these solutions was measured, and showed in Fig. 5. Since even the intact α-amylase in aqueous solution accepts inactivation spontaneously to some extent, the vertical axis in Fig. 5 shows the remaining activity ratio against the activity of none irradiated enzyme at each time. The irradiated enzyme did not only accept further inactivation in this incubation time, but recovered its activity a little.

On the contrary, when the irradiated enzyme solution was diluted to 1/1000 of its original and incubated at 4°C, the activity was decreased remarkably.

DISCUSSION

SH enzyme had been considered to be more sensitive to irradiation by many workers, but it seems that the difference of sensitivity to irradiation between SH enzymes and non SH enzymes is not so large. For example, D_{37} of urease[7] and liver dehydorgenase,[8] known to be SH enzymes, are about 9×10^3 rad irradiating in the 1.0 percent former solution and 10×10^4 rad irradiating in the 0.09 percent latter solution respectively.

D_{37} of B.S. proteinase[9] irradiated in 0.125 percent solution and B.S. α-amylase irradiated in 0.1 percent solution, both are non SH enzymes, are 7×10^4 rad and 40×10^4 rad respectively. Furthermore, these values should have been influenced by the purity or coexistent substances in the enzyme preparations.

These effects on the inactivation rate of α-amylase will be described elsewhere. From these facts α-amylase seems not to be so resistant, non to be so sensitive to the irradiation treatment. Concentration effect shown in Fig. 2 indicates that the inactivation mechanis of this enzyme in aqueous solution is indirect action. Though many species of radiolyzate are known to be produced from water by irradiation, at least hydrogen peroxide, one of such substances, does not play an important part for the inactivation of this enzyme. The amount of hydrogen peroxide required for reducing

the enzyme activity in 0.1 percent enzyme solution to 40 percent of original one was as much as 0.3 M.

But by the irradiation of 8×10^4 rad, this enzyme in 0.1 percent solution was inactivated to the same extent and the amount of hydrogen peroxide produced from water with this dose was only 0.57×10^{-4} M, because of its G-value 0.71[10]. Therefore the effect of hydrogen peroxide in the inactivation by irradiation could be neglected. On the contrary, when the enzyme solution was irradiated with UV lights in the presence of hydrogen peroxide, its activity was reduced rapidly. As the dose measurement of UV lights irradiated was impossible, the amount of OH radical produced photochemically was unknown. But considering the fact that either addition of H_2O_2 or the irradiation of UV lights have little effect on the inactivation, it can be concluded that the inactivation of enzyme is chiefly caused by the action of free radical OH• which has a very short life time. The fact that the irradiated enzyme did not accept further inactivation after irradiation may be interpreted as the little effect of hydrogen peroxide on this enzyme.

The inactivation of irradiated enzyme in diluted solution would be caused by the reduction of its stability.

As Fig. 1-b shows the one hit type inactivation, this enzyme molecule is presumed to have some important site or comformation to maintain its activity.

ACKNOWLEDGMENT

The author would like to express her sincere thanks to Professor Sakae Shimizu for his arrangement to use the Irradiation Facility of the Institute for Chemical Research, Kyoto Univ., and to Professor Hiroyuki Hatano and Dr. Koichiro Sumizu for their kind direction and discussion and also thank the Daiwa Kasei Co. Ltd., Osaka, for the kind supply of the crystslline α-amylase.

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